REVERSIBLE, PARALLEL AND MULTITASK CLONING METHOD AND KIT

Field of the invention

[0001] The present invention is related to a reversible, parallel and/or multitask cloning method and kit, which improves the cloning of (preferably multiple) genetic element(s) in a nucleic acid construct such as a vector or the chromosome of a cell and the rapid and efficient selection of constructs with a correct integration of said genetic element(s), either in vitro or in vivo.

Background of the invention

[0002] To obtain complex molecular constructs comprised of multiple genetic elements, the selection of the genetic events (insertion(s) and/or deletion(s) and/or inversion(s) of DNA fragments) that will cause the assemblage of the target construct comprised of the said genetic elements at the right position and with the right orientation is usually a time consuming procedure.

[0003] In particular, one is necessary faced with the major problem of selecting different multiple genetic events (insertion, deletion, inversion of a genetic sequence in a nucleic acid construct), possibly in the same reaction tube.

[0004] Therefore, a molecular biologist should usually obtain a genetic event (insertion, deletion, inversion of a genetic sequence in a nucleic acid construct) separately and not simultaneously in the same

reaction tube and should avoid any mistake (incorrect integration of a genetic sequence in the wrong direction, etc,) during said genetic manipulation.

Aims of the invention

[0005] A first aim of the invention concerns methods and tools which provide a solution to the above-mentioned problems, in particular methods and tools which allow a molecular biologist to insert and/or remove a genetic element, or to obtain a modification in the lecture orientation of said genetic element (inversion) in a nucleotide sequence, either in vitro or in vivo.

[0006] Another aim of the present invention is to provide methods and tools which allow the creation of a genetic construct (such as a vector or the chromosome of a cell), either in vitro or in vivo, and assembled through the insertion(s), deletion(s) and/or inversion(s) of multiple genetic elements and the selection of the said genetic construct having incorporated (deleted or inverted) correctly these genetic elements.

[0007] A further aim of the present invention is to provide tools which allow a biologist to perform the step of the method in parallel and to perform at the same time multiple tasks (selection of multiple genetic events) in the same reaction tube or not.

[0008] A last aim of the present invention is to provide tools which allow that genetic events (insertions and deletions and inversions) are reversible, such that any nucleic acid construct can be viewed as a set of elements that can be recycled, i.e., re-used for the assemblage of other different nucleic acid constructs.

Summary of the invention

[0009] In the method and kit described hereafter, the person skilled in the art uses specific genetic constructs, which are the tools for performing the cloning and selection method according to the invention. Said tools are genetic constructs that could be integrated in vector(s) (plasmid(s) or virus(es), including bacteriophage(s)) or in the chromosomal genome of a cell suitable for obtaining the cloning and selection of the correct assemblage of various genetic elements. All these methods and systems allow the assemblage of one or more foreign genetic element(s) (target sequences of interest) in said nucleic acid construct vector or chromosome of a cell at specific sites. The integration of a foreign (preferably autologous) genetic element the nucleic acid construct of the invention could be done by techniques known to the person skilled in the art such as, but not limited to classical restriction/ligation, site specific recombination, TOPO cloning and homologous recombination. The assemblage of genetic elements can involve insertion(s), deletion(s) and/or inversion(s) of nucleotide sequences. In the method according to the invention, the selection of correctly inserted sequences is obtained by using specific markers, which are nucleotide sequences encoding molecules that are toxic for a cell or molecules which are inhibitors of such toxic molecules and/or block to toxic activity of such molecules expressed in the cell. Preferably, said molecules are either poison(s), and/or inhibitor(s) to poison(s), preferably selected from (but not restricted to) the group consisting of the following poison/antidote systems: Ccdb/Ccda, Kid/Kis, Hok/Sok, Doc/Phd, RelE/RelB, PasA/PasB/PasC, MazE/MazF, ParE/ParD.

[0010] In the method according to the invention, said foreign nucleotide elements are advantageously linked

(at its 3′ or 5′ or both ends) to one or more promoter/operator nucleotide sequences, such as, but not limited to, constitutive promoters allowing the expression of a target nucleotide sequence incorporated in the nucleic acid construct according to the invention, when they are disposed according to the suitable and requested lecture orientation.

[0011] In the method according to the invention, the person skilled in the art uses suitable cell strain(s) (prokaryotic and/or eukaryotic) which are either resistant or sensitive to one or more of said toxic molecules in order to obtain and select recombinant(s). The properties of cell strains can for example be due to the existence of gene(s) coding for poison and/or antidotes and integrated in the chromosome(s) of a cell or presented in episomal sequences such as plasmids.

Reversible cloning and selection method and kit

[0012] A first aspect of the present invention is related to a reversible cloning method and kit for which several specific preferred examples are described in details hereafter, in reference to the figures 2 to 5.

[0013] The elements used in the method of the invention are specific cells and a genetic preferably integrated in a vector or a chromosome of a cell comprised of either :

- a promoter/ activator sequence 11 disposed upstream of a first and a second nucleotide sequence (1,2) encoding two different toxic molecules (such as a poison 1 and a poison 2) (figure 2, left), or
- a first promoter/activator sequence 11 disposed upstream of a first nucleotide sequence 1 encoding a toxic molecule (such as a poison 1) and, disposed in

the opposite lecture direction of the first promoter/activator sequence 11, a second promoter/activator sequence 12 disposed upstream of a second nucleotide sequence encoding an antidote 2' to a second toxic molecule (such as poison 2) (figure 3, left), or

- a promoter/activator sequence 11 disposed upstream of a first and a second nucleotide sequence (1,2') encoding, respectively, a first toxic molecule (such as poison 1) and an antidote to a second toxic molecule (such as poison 2) different from said first toxic molecule (figure 4, left).
- The terms "a nucleotide sequence encoding a toxic molecule or an antidote to a toxic molecule" also include sequences comprising multiple coding portions encoding several identical toxic molecules.

[0014] The insertion of a foreign target nucleotide sequence (A) "in" or as "a replacement" of the nucleotide sequence (1) encoding a toxic molecule element will allow either :

- the inactivation of the nucleotide sequence 1 encoding the first toxic molecule, plus the activation or maintenance of the activation of the sequence 2 encoding the second toxic molecule (figure 2); or :
- the inactivation of the first nucleotide sequence 1 encoding the first toxic molecule, plus the inactivation of the nucleotide sequence 2 encoding the antidote to the second toxic molecule (figure 3); or :
- the inactivation of the first nucleotide sequence 1 encoding the first toxic molecule (figure 4).

[0015] The inserted foreign genetic element(s) (target sequence) may be a regulatory sequence or gene(s)

of interest (possibly linked to one or more promoter/operator sequences).

[0016] The selection of the genetic event (insertion) can be obtained in a cell strain sensitive to the first toxic molecule 1 (Figs. 2&3&4) and possibly resistant to the second toxic molecule 2 (Fig. 2).

[0017] However, the said genetic event (insertion or replacement) is reversible through the replacement of the inserted element (target sequence) by the element that had been deleted following the recombination and insertion made in the first step. This reverse reaction deletion of a target sequence is selected in a strain both resistant to the toxic molecule 1 and sensitive to the toxic molecule 2 (Figs 2, 3, 4) plus, possibly, producing the toxic molecule 2 (Figs. 3&4).

[0018] This reversible cloning and selection method is also suitable for obtaining an inversion of an integrated genetic element. A specific example is described in details hereafter, in reference to the figure 5. Indeed, the orientation of a sequence of interest can be reversed through the method of the invention (preferably following the insertion step of figure 4) or through a direct insertion of the target sequence between two different antidote sequences (1', 2'). Said genetic element (target sequence) associated to a promoter/operator (either at its 3' or 5' end), is initially integrated between two nucleotide sequences (1',2') encoding respectively two different antidotes to two different toxic molecules 1 and 2. Said two nucleotide sequences (1',2')encoding the two different antidotes are disposed in opposite lecture orientations (disposed upstream and downstream the target nucleotide sequence in opposite divergent lecture orientation). This construct allows to select for the recombination event(s) which will cause the target

nucleotide sequence of interest and its associated promoter to have either the same orientation as the nucleotide sequence 1' encoding the first antidote to the first toxic molecule (selection done in a strain both sensitive to and producing poison 1) or the same orientation as the nucleotide sequence 2' encoding the second antidote to the second toxic molecule (selection done in a strain both sensitive to and producing poison 2). (see WO 02/066657 incorporated herein by reference)

Parallel and/or multitask cloning and selection

[0019] The above-mentioned reversible cloning and selection method and elements (nucleic acid construct or vector and specific cells strains) can also be used in a parallel and/or multitask cloning and selection method described hereafter (in details in the following example in reference to the figure 1).

[0020] The assemblage of multiple foreign genetic elements (different target sequences) in the vector or in the chromosome of a cell (either in vitro or in vivo) and the selection of the correct assemblage is obtained by the use of multiple nucleic acid construct comprising sequences encoding one or more (different or identical) toxic molecules and/or their antidotes. According to the type of the nucleic acid construct and the type of selective markers (encoding toxic molecule(s) and/or antidote(s) to toxic molecule(s)), the person skilled in the art can select the suitable events of insertion(s), deletion(s) and/or inversion(s) applied with said multiple genetic element(s).

[0021] Said cloning and selection method may require multiple steps possibly performed (sequentially) in the same reaction tube or inside a single cell.

[0022] Said method can be combined with the steps and means for performing in vitro protein synthesis (using in vitro transcription and translation kits).

[0023] Another aspect of the present invention is related to the algorithms, computer programs, and data bases (comprised of codes and means possibly stored in a computer readable medium) that can assist performing one or more step(s) of the method according to the invention. Said algorithms, data bases, and program codes means are used to define the correct combination of (but not limited to) :

- suitable markers (encoding the toxic molecule and/or the antidotes to said toxic molecule);
- suitable cell strain(s) for selecting the suitable genetic events;
- suitable pre-starting nucleic acid construct(s),
- suitable genetic element(s) (target nucleotide sequences and/or their operator/promoter sequences) to be inserted, deleted and/or reversed;
- reaction mixture (including but not restricted to recombines mixtures, buffer, media, enzymes,...) that are necessary for the assemblage/production of the molecular construct.

[0024] The algorithms, computer programs, and data bases are also able to control one or more step(s) of the method according to the invention, possibly performed by automate(s).

[0025] Another aspect of the present invention is related to kits of parts (cloning and/or selection kits) comprising the suitable elements for performing the method according to the invention, in particular computer programs mentioned above, nucleic acid construct(s), cell strain(s)

and/or usual products and media used in the cloning and selection techniques.

[0026] Another aspect of the present invention is related to automates allowing to perform the method according to the invention and using the above-mentioned kit(s) of parts. Said kit(s) of parts (cloning and selection kits, combined with adequate media, cells and media present in vitro transcription and translation kits) and automates could also comprise other elements, such as a buffer solutions, pipeting element(s), primers for genetic amplification, cell culture media and means for recording results and for the storage of data.

[0027] The present invention will be described in detail in the following examples, in reference to the enclosed figures presented as non-limiting illustration of the various aspects of the present invention.

Short description of the drawings

[0028] The figure 1 is an example of complex genetic construct obtained by parallel and multiple genetic events performed by the method of the present invention.

[0029] The figures 2 to 5 are examples of reversible cloning and selecting method and kit according to the invention.

Detailed description of the invention

[0030] This invention allows the making of complex genetic constructions through the use of *(i)* simultaneous and *(ii)* parallel events (the various recombinations and selection events present almost the same frequency). The "multitask" nature of the invention is defined as follows: for example, the invention allows to perform the insertion of genetic elements *A* and C, the deletion of genetic elements *E* and *F*, and the inversion of genetic elements *B*

and *D*, some or all events (Fig.1) being performed simultaneously *in vitro* (*i.e.*, in the same tube) or *in vivo* (*i.e.*, in the same organism). The final product of the above-mentioned events is a complex construct comprised of the genetic elements *A*, *B*, *C*, and *D*, all with the same orientation. The simultaneous selection of several genetic events (*e.g.*, here, insertions, deletions, inversions, recombinations) is achieved through the use of a different selective marker (here poisons and antidotes genes for example) for each of the events. Filled black arrows represent promoters.

[0031] Plasmid 1 is amplified in a strain resistant to poison 1. Plasmid 2 is amplified in a strain resistant to poisons 6 and 9. Plasmid 3 is selected in a strain :

- sensitive to poisons 1 and 6 (for the selection of the insertion of genetic elements A and C),
- sensitive to poisons 3 and 5 (for the selection of the inversions of genetic elements B and D),
- sensitive to poisons 7 and 8 (for the selection of the deletions of genetic elements E and F),
- sensitive to poison 9 (for the selection of the recombination event between the construct made from plasmid 1 and the construct made from plasmid 2),
- and producing poison 3, poison 5, poison 7 and poison 8.

[0032] Realization of each "recombination" event can be done through techniques such as, but not limited to, classical restriction/ligation, site-specific recombination, or homologous recombination. Specificity of each genetic event (insertion, deletion, inversion, etc.) is insured by the specificity of the recombination event. For example, specificity of an insertion (both the location of the insertion and the orientation of the insert (target

nucleotide sequence)) can be achieved by the use of different DNA sequences bordering both the insertion site and the fragment to be inserted (these DNA sequences can be selected by the man skilled in the art for performing said recombination event). These flanking sequences form either different site-specific recombination elements (in the case of site-specific recombination) or different elements of homology (in the case of homologous recombination). The simultaneous selection of several genetic events (*e.g.*, an insertion, a deletion, and an inversion) is achieved through the use of a different selective marker for each of the events. As each of the genetic events is rare by nature, the selection for the simultaneous presence of all events requires the use of very efficient selective markers (*e.g.*, but not limited to, antidote/poison genes).

[0033] The parallel cloning nature of the invention is defined as follows: N different genetic constructs that are produced in the same reaction mix (*i.e.*, in the same tube) through the multitask process described above can be pre-designed such that their assemblage (here, the assemblage of the construct made from plasmid 1 with the construct made from plasmid 2) can be generated through recombination events as well. In other words, N-1 genetic constructs can be viewed as donors and 1 construct as a receptor. For example, N constructs can be combined through the use of n-1 selective markers for the selection of n-1 recombination events (Fig. 1).

[0034] Furthermore, the invention allows to use the products of the multitask/parallel cloning process as building blocks of new reactions. Indeed, a construct produced through the invention is a unique combination of building blocks that can be re-used for new (and different) constructs; *i.e.*, the process is reversible and extendable, as shown in the Figures. 2 to 4.

[0035] In figure 2, the DNA fragment to be inserted encodes for a target sequence of interest plus a promoter sequence located at its 3' end. The nucleic acid construct comprising the adequate insertion is selected by the deletion of the nucleotide sequence 1 encoding poison 1 in a strain sensitive to it but resistant to poison 2. The deletion of the target sequence (DNA fragment A) for re-use of the building block is achieved through the insertion of the DNA fragment initially removed (i.e., nucleotide sequence 1 encoding poison 1 with a promoter at its 5' end). This reverse event is selected in a strain sensitive to poison 2 and resistant to poison 1. Plasmid 1 is amplified in a strain resistant to poison 1. Plasmid 2 is amplified in a strain resistant to poison 2.

[0036] In figure 3, the insertion of the target sequence (DNA fragment A) is selected by the deletion of the nucleotide sequence 1 encoding poison 1 in a strain sensitive to it. The deletion of the DNA fragment A for re-use of the building block is achieved through the insertion of the DNA fragment initially removed (i.e., poison 1 with, at its 5' end, two promoters in opposite directions). This reverse event is selected in a strain allowing the conditional expression of poison 2, sensitive to poison 2, and resistant to poison 1. Plasmid 1 is amplified in a strain resistant to poison 1. Plasmid 2 is amplified in any strain whose viability is independent from the presence or absence of plasmid 2.

[0037] In figure 4, Plasmid 1 encodes both poison 1 and antidote 2 that are organized as an operon. The insertion of the target sequence (DNA fragment A) is selected by the deletion of the nucleotide sequence encoding poison 1 in a strain sensitive to it. The deletion of the target sequence (DNA fragment A) for re-use of the building block is achieved through the insertion of

the DNA fragment initially removed (i.e., nucleotide sequence encoding poison 1 with a promoter at its 5' end). This reverse event is selected through the activation of the nucleotide sequence encoding antidote 2 in a strain allowing the conditional expression of poison 2, sensitive to poison 2, and resistant to poison 1. Plasmid 1 is amplified in a strain resistant to poison 1. Plasmid 2 is amplified in any strain whose viability is independent from the presence or absence of plasmid 2.

[0038] In figure 5, the target sequence (DNA fragment A) contains a promoter allowing the production of an antidote. The inversion of the DNA fragment A is selected using a strain allowing the conditional expression of poison 2 and sensitive to it. The reverse event is selected in a strain allowing the conditional expression of poison 1 and sensitive to it.

[0039] In other words, constructs produced through the invention are not dead-end products (*i.e.*, useful for the only use they have been produced for); they can be recycled. This emphasizes the importance of the software component of the invention because it allows to create not only a data base of building blocks, but also of products that are followed up and stored (virtually in computers, and physically in freezers or other devices) for potential future uses. Because the software tracks the features of each building block and product, it also identifies those elements that are *(i)* necessary and *(ii)* inter-compatible for future and new multitask/parallel/reversible processes.